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Light is Essential for Degradation of Ribulose-1,5-Bisphosphate Carboxylase-Oxygenase Large Subunit During Sudden Death Syndrome Development in Soybean

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Abstract: *Fusarium solani* f. sp. *glycines* (Fsg) has been reported to produce at least two phytotoxins. Cell-free Fsg culture filtrates containing phytotoxins have been shown to develop foliar sudden death syndrome (SDS) in soybean. We have investigated the changes in protein profiles of diseased leaves caused by cell-free Fsg culture filtrates prepared from Fsg isolates. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was conducted to investigate the protein profiles of diseased and healthy leaves. An approximately 55 kDa protein was found to be absent in diseased leaves. Matrix-assisted laser desorption-ionization time-of-flight mass spectrometric analyses and a database search revealed that the missing protein is the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit, which is involved in carbon assimilation and photorespiration. This result was confirmed by Western blot experiments. We have shown that light is essential for disappearance of the Rubisco large subunit initiated by cell-free Fsg culture filtrates. The disappearance of the protein is fairly rapid and occurs within 24 h, presumably due to degradation. Cell-free, Fsg culture-induced degradation of the Rubisco large subunit was accompanied by accumulation of reactive oxygen species under light conditions. Terminal deoxynucleotidyl transferase-mediated nick end labelling experiments suggested that programmed cell death was initiated in leaves of seedlings fed with cell-free Fsg culture filtrates. These results suggest that, in the presence of light, Fsg culture filtrates containing phytotoxins cause degradation of the Rubisco large subunit and accumulation of free radicals and, thereby, initiate programmed cell death leading to foliar SDS development in soybean.

Key words: *Fusarium solani* f. sp. *glycines*, *Fusarium virguliforme*, soybean, SDS, Rubisco, MALDI-TOF-MS, TUNEL.

Abbreviations:

Fsg: *Fusarium solani* f. sp. *glycines*
 SDS: sudden death syndrome
 PAGE: polyacrylamide gel electrophoresis
 Rubisco: ribulose 1,5-bisphosphate carboxylase/oxygenase

MALDI-TOF-MS: matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
 TUNEL: terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling

Introduction

Sudden death syndrome (SDS) of soybean is caused by the fungal pathogen, *Fusarium solani* f. sp. *glycines* (Fsg). The fungus has recently been renamed *Fusarium virguliforme* (Aoki et al., 2003). SDS has been detected in Argentina, Brazil, and the USA (Rupe and Hartman, 1999). In the United States, the disease has been reported in more than ten states and the estimated average annual crop loss from the disease has been valued at around US\$ 100 million (Wrather et al., 2001). SDS development is affected by environmental factors such as rainfall, soil fertility, and planting date. It is also affected by genotype and soybean cyst nematode infection (Rupe et al., 1991, 1993; Chang et al., 1997).

SDS is characterized by root rot, crown necrosis, premature defoliation, and flower and pod abortion (Rupe, 1989). Fsg is a soil-borne fungal pathogen (Roy et al., 1997). In susceptible soybean cultivars, the infected roots show crown necrosis and rotting symptoms within 2 weeks of planting. Fsg produces a large number of asexual spores on the surface of the severely infected soybean roots. SDS foliar symptoms, also known as leaf scorch, develop slowly. About 1 or 2 months following planting, interveinal chlorotic spots appear in infected susceptible plants. The spots develop into necrotic or chlorotic streaks and, in the late stage, premature defoliation occurs (Melgar and Roy, 1994). Fsg has never been isolated from aboveground portions of the soybean plant. The foliar symptoms are therefore presumably induced by Fsg phytotoxins (Roy et al., 1989; Rupe, 1989; Strobel, 1982). Under greenhouse conditions, leaf scorch resistance is governed by a single gene in the cultivar, Ripley, and by two genes in P9451 (Stephens et al., 1993; Ringler and Nickell, 1996). Under field conditions, SDS resistance is partial and governed by multiple genes (Chang et al., 1996, 1997; Hnetkovsky et al., 1996; Meksem et al., 1999; Njiti et al., 1996, 1997, 2002). Several quantitative trait loci for SDS resistance have been identified (Iqbal et al., 2002; Njiti et al., 2002).

In plants, programmed cell death (PCD) is initiated during development, senescence, exposure to phytotoxins and under stresses caused by high ozone concentration and pathogen attack (Buchanan et al., 2000; Greenberg, 1996; Yao et al., 2001; Pasqualini et al., 2003; Levine et al., 1996). There are several host-selective toxins that trigger PCD leading to necrosis (Wolpert et al., 2002). For example, victorin, a toxin produced by *Cochliobolus victoriae*, causes a mitochondrial oxidative burst and PCD in oat (Yao et al., 2001; Navarre and Wolpert, 1999). AAL toxin produced by *Alternaria alternata* f. sp. *lycopersici* causes PCD and the *Alternaria* stem canker disease in tomato (Lincoln et al., 2002). AM toxins I–III, produced by the *Alternaria alternata*, are host-specific. AM toxins inhibit CO₂ fixation and cause leaf necrosis. Chloroplasts are the primary target of AM toxins that cause leaf spot disease in apple (Miyashita et al., 2003). The wheat pathogen, *Pyrenophora tritici-repentis*, secretes at least two host-selective toxins, ToxA and ToxB, encoded by single genes. A recent study has indicated that in toxin-sensitive wheat lines a host factor(s) is involved in internalization of ToxA into the cytoplasm and chloroplasts (Manning and Ciuffetti, 2005). The toxin causes cell death in both toxin-sensitive and insensitive wheat lines if expressed through transformation procedures. The mechanism used by the toxin to cause disruption in the thylakoid structure is, however, not yet known.

Light is known to play an important role in activating phytochemicals. Cercosporin is maintained in inactive form in fungi but, after being released from the fungal hyphae, it is oxidized to acquire photodynamic activity (Daub et al., 1992). *Cercospora* toxins, Ceбетin A and B inhibit the plasma membrane and chloroplast ATPases of sugar beet in a light-dependent manner (Jalal et al., 1992). The *F. solani* toxin, dihydrofusarubin, causes the degradation of tobacco leaf pigments in a light-dependent manner (Heiser et al., 1998, and references therein). In spinach, dihydrofusarubin causes the formation of reactive oxygen species (ROS), such as superoxide, by interrupting the photosynthetic electron transport chain of chloroplasts (Albrecht et al., 1998).

Tentoxin, produced by *Alternaria alternata*, inhibits photophosphorylation by binding to the chloroplast ATPase. This leads to decreased CO₂ fixation and an increased number of free electrons that cause ROS generation (Pinet et al., 1996). Tabtoxin, produced by *Pseudomonas syringae* pv. *tabaci*, causes chlorotic lesions in tobacco leaves in a light-dependent manner (Durbin, 1981). Rubisco carboxylation activity is inhibited and, as a result, ATP and NADPH generated from photophosphorylation are not utilized for carbohydrate assimilation and ADP and NADP⁺ are not generated for further photophosphorylation. This leads to ROS generation through transfer of electrons from photosystem I to molecular oxygen rather than to ADP or NADP⁺ (Heiser et al., 1998, and references therein).

ROS produced by phytochemicals can cause peroxidative breakdown of unsaturated fatty acids. Chlorophyll is oxidized and bleached by alkoxyl radicals produced from the peroxidation of fatty acid (Heiser et al., 1998). For example, cercosporin, produced by *Cercospora* sp. *cercospori*, causes lipid peroxidation (Elstner and Osswald, 1980; Elstner et al., 1985; Heiser et al., 1998). *F. solani* naphthazarin toxins are electron acceptors for reducing oxygen during formation of ROS. These toxins enhance membrane permeability (Medentsev et al., 1988; Ne-

mec, 1995) and cause chlorosis of citrus leaves. They disrupt chloroplasts by causing chloroplast membranes to swell and by disorganizing the granule stacks (Achor et al., 1993; Nemec, 1995).

Cell-free culture filtrates of *Fsg* have been found to cause SDS foliar symptoms routinely in 3-week-old seedlings and it was suggested that *Fsg* phytochemicals translocated from roots are responsible for development of foliar symptoms (Li et al., 1999). The cell-free *Fsg* culture filtrates can also cause cell death (Li et al., 1999). Cell-free culture filtrates isolated from non-pathogenic *F. solani* isolates did not cause leaf scorch in soybean (Li et al., 1999). Most likely, *Fsg* toxins produce leaf scorch only in soybean. Two phytochemicals have been isolated from the cell-free *Fsg* culture filtrates: monorden and a 17-kDa proteinaceous toxin. Purified fractions including the 17-kDa protein cause browning of soybean calli and necrosis in detached soybean leaves (Baker and Nemec, 1994; Jin et al., 1996). The gene encoding this proteinaceous toxin has not been isolated. The role of the toxin in leaf scorch development is also unknown. We have investigated changes in protein profiles that occur during leaf scorch development following feeding of soybean seedlings with cell-free *Fsg* culture filtrates. We have observed that the Rubisco large subunit is degraded in diseased leaves. Light is essential for the degradation of Rubisco large subunit by the cell-free *Fsg* culture filtrates. Data presented in this paper suggest that, in the presence of light, *Fsg*-specific toxin(s) causes degradation of Rubisco large subunit and accumulation of free radicals, which presumably cause PCD and leaf scorch development in soybean.

Materials and Methods

Preparation of fungal culture filtrates

Fusarium solani f. sp. *glycines* isolates Clinton and Scott were grown and maintained on solid Bilay medium (0.1% KH₂PO₄ [w/v], 0.1% KNO₃ [w/v], 0.05% MgSO₄ [w/v], 0.05% KCl [w/v], 0.02% starch [w/v], 0.02% glucose [w/v], and 0.02% sucrose [w/v]) for 12 days at 23 °C in the dark. Forty plugs were transferred into 100 ml modified Septoria medium (MSM) and incubated for 12 days without shaking at 23 °C in the dark (Song et al., 1993). Cultures were filtered through two layers of Whatman No.1 filter paper and the pH was adjusted to 6.0 with HCl. The cultures were refiltered through a 0.45-mm stericup and then through a 0.22-mm stericup (Millipore, Inc.) and the cell-free *Fsg* culture filtrates were stored at 4 °C until use. Filtered MSM was used as the control (Li et al., 1999).

Stem cutting assay

The soybean cultivar Williams 82 was grown in soil for 3 weeks to second trifoliate stage in a growth chamber under a 16-h photoperiod at 200 µmol photons/m²/s and 25 °C, and in darkness at 16 °C. Cell-free *Fsg* culture filtrates were diluted in sterile, double distilled water. Soybean seedlings were cut below the cotyledon. Cut seedlings were placed in 50 ml Oakridge tubes containing 25 ml diluted, cell-free *Fsg* culture filtrate or diluted MSM filtrate as a control (Li et al., 1999).

Leaf protein extraction

One g of leaf tissue was ground in liquid nitrogen. The ground tissue powder was then mixed with 5 ml chilled acetone containing 10% trichloroacetic acid (TCA) (w/v) and 0.07% β -mercaptoethanol (β -ME) (v/v) and kept at -20°C for 1 h. The samples were then centrifuged at 13 000 rpm for 10 min and the insoluble material was washed in ice-cold acetone containing 0.07% β -ME (v/v). The pellets were kept at -20°C for 20 min and then centrifuged at 13 000 rpm and 4°C for 10 min. The washing, incubation, and centrifugation steps were repeated twice. The pellets were vacuum-dried in a Speed Vac (Savant Instruments, Inc., NY) for 3 min. One ml rehydration buffer (7 M urea, 2 M thiourea, 2 mM tributyl phosphate, 4% [w/v] CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate], 0.5% ampholytes) was added to solubilize the proteins. Tributyl phosphate and ampholytes were added just before use.

Two-dimensional electrophoresis

Electrophoresis of protein samples was conducted in a 24-cm immobilized pH gradient (IPG) gel (pH 4.0–7.0) at 100 V for 4 h followed by 500 V for 4 h, 1000 V for 6 h, 4000 V for 6 h, and 8000 V for 20 h in an Ettan IPGphor system (Amersham Biosciences, NJ). Subsequently, proteins were separated in the second dimension on a 12% sodium dodecyl sulfate-polyacrylamide gel at a constant current of 40 mA in an Ettan DALTsix large vertical electrophoresis system (Amersham Biosciences, NJ). The second-dimension gels were stained with modified Coomassie Blue stain (Coomassie Blue G-250 0.1% [w/v], methanol 34% [v/v], phosphoric acid 3% [v/v], ammonium sulfate 10% [w/v]), for at least 24 h. Stained gels were destained with 1% (v/v) acetic acid for at least 24 h. A cluster of protein spots, absent in protein samples prepared from diseased leaf tissues showing moderate symptoms (interveinal chlorosis and necrosis), were picked for identification.

Protein in-gel digestion

A cluster of protein spots and a 55-kDa protein band, not present in leaf scorch diseased tissues, were identified on a two-dimensional (2-D) sodium dodecyl sulfate-polyacrylamide gel and single dimension (1-D) sodium dodecyl sulfate-polyacrylamide gel of healthy tissues, respectively, and isolated using a 20-gauge needle. The gel pieces were transferred into 400 μl soaking buffer (50% [v/v] acetonitrile in 25 mM ammonium bicarbonate, pH 8.0) in a 500 μl microcentrifuge tube. The gel fragments were soaked in the buffer for 15 min. The supernatant was removed and the gel pieces were washed with 400 μl soaking buffer three more times. Finally, the gel fragments were soaked in 100% acetonitrile for 5 min. The acetonitrile was then removed and gel fragments were dried for 20 to 30 min in a Speed Vac (Savant Instruments, Inc., NY). The gel fragments were rehydrated with a minimal volume of trypsin solution (10 μg of trypsin in 1 ml of 25 mM ammonium bicarbonate buffer, pH 8.0) (Promega, Inc., WI). The gel was incubated at 37°C for 16 to 24 h. After removal of the supernatant, gel fragments were soaked in and extracted with 25 μl aliquots of 50% acetonitrile (v/v)/5% trifluoroacetic acid (v/v) for 30 to 60 min. Extracts were dried in a Speed Vac (Savant Instruments, Inc., NY) to reduce the volume to 5 to 10 μl .

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS)

A 1 μl digested protein sample and 1 μl of matrix (α -cyano-4-hydroxycinnamic acid [ACH], 10 mg/ml) were mixed thoroughly. One μl of this mixture was loaded onto the sample target of a Dynamo MALDI mass analyzer (Thermo Bioanalysis, Ltd, UK). After the mixture was dried, a 0.5- μl calibration standard was loaded onto the sample target spot. Voyager control software (Applied Biosystems, Inc., CA) was used to eject the sample and obtain the mass spectrum of the tryptic peptides. To identify the protein, masses of tryptic peptides determined by MALDI-TOF were compared with the calculated peptide mass fingerprints of the protein sequence databases (NCBI nr.10.21.2003) with the aid of the program MS-Fit (<http://prospector.ucsf.edu/ucsfhym13.4/msfit.htm>).

Free radical assay

The nitro-blue tetrazolium (NBT) staining method was used to detect O_2^- accumulation in leaves. Leaves were vacuum-infiltrated with 0.5 mg/ml NBT solution for 5 min three times. Then leaves were incubated in the NBT solution in darkness for 1 h at room temperature. After removal of the NBT solution, leaves were incubated at 50°C in 80% ethanol until chlorophyll was completely bleached (Duttilleul et al., 2003). The 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining method was applied to detect H_2O_2 accumulation in leaves. Leaves were vacuum-infiltrated with DAB solution (1 mg/ml) for 5 min for three times. Then leaves were incubated in the DAB solution in darkness for 14 h at room temperature. After removal of the NBT solution, leaves were incubated at 50°C in 80% ethanol until the chlorophyll was completely bleached (Duttilleul et al., 2003).

Western blotting

Fifteen μg of total protein from each sample were separated in a 12% polyacrylamide-sodium dodecyl sulfate mini gel. Following electrophoresis, gels were electro-blotted onto a PVDF membrane (Millipore, Inc., MA). The membrane was blocked with 1% bovine serum albumin (BSA). The membrane was incubated with anti-Rubisco large subunit antibody (1 : 2000 dilution: AgriSera, Inc., Sweden) or anti-GDC-P protein antibody (1 : 1000 dilution) for 3 h. Anti-chicken IgY alkaline phosphatase conjugate (Promega, Inc., WI) and anti-rabbit IgG b + l alkaline phosphatase conjugate (Bethyl Laboratories, Inc., TX) were used as the secondary antibodies for anti-Rubisco large subunit antibody and anti-GDC-P protein antibody, respectively. The membranes were incubated in Western Blue Stabilized Substrates (Promega, Inc., WI) for detecting alkaline phosphatase activity.

In situ cell death detection: terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) staining

Soybean leaves showing early symptoms (chlorotic spots) were collected from cell-free Fsg culture filtrate-treated seedlings. Leaves collected from MSM-treated seedlings were used as the negative control. Circular leaf sections 0.6 cm in diameter were punched from the collected leaves and fixed in freshly prepared 4% paraformaldehyde and 0.25% glutaraldehyde (v/v) in 0.1 M phosphate buffered saline (PBS) (pH 7.4) over-

night at 22°C. Leaf tissues were then washed in PBS buffer for 30 min. Leaf tissues were dehydrated in 70 to 100% ethanol for 3 h, treated with xylene and embedded in paraffin. Leaf tissues were cross-sectioned to 10 µm thickness and de-waxed. Sections were treated with proteinase K for 20 min at 23°C. Fifty µl of TUNEL reaction solution (5 µl terminal deoxynucleotidyl transferase [TdT] and 45 µl of labelled nucleotides from an *in situ* cell death detection kit [Roche Applied Science, Inc., IN]) were added to label the free 3'-OH group of the broken end of DNA molecules in a template-independent manner. Fifty µl of TUNEL reaction solution were added to MSM-fed leaf tissues to serve as a negative control and 10 units of DNase were added to MSM-fed leaf tissues to serve as a positive control. The tissues were incubated at 37°C in a water bath in darkness for 60 min. Then leaf tissues were washed in PBS buffer three times. The fluorescent spots were visualized with a fluorescence microscope using a green filter (515–565 nm wavelength). Nuclei were localized by staining the tissue preparations with 0.2 µg/ml Hoechst 33258 dye and observed under a fluorescence microscope (Carl Zeiss, Inc., Germany) using a blue filter (352–461 nm wavelength).

Results

Cell-free *Fsg* culture filtrates cause foliar SDS symptoms

Earlier, it was shown that symptoms of foliar SDS were induced when 3-week-old seedlings were fed with cell-free *Fsg* culture filtrates through cut stems (Li et al., 1999). This assay, termed the stem cutting assay, produces highly reproducible foliar symptoms as compared to those caused by root inoculation with *Fsg* spore suspensions. Two *Fsg* isolates, Clinton and Scott, isolated and kindly provided by X.B. Yang, produce similar disease symptoms on the susceptible soybean cultivar, Williams (Fig. 1). The colour of culture medium containing the Clinton isolate becomes reddish after about 12 days of growth, and the production of reddish colour is associated with the development of foliar SDS. Therefore, we chose to use the Clinton isolate in this investigation.

Steady-state levels of Rubisco large subunit were decreased in diseased tissues

The total soluble protein profiles of healthy and diseased leaves were compared using 2-D PAGE (Fig. 2). A cluster of abundant proteins was consistently absent in diseased tissues (Fig. 2B). MALDI-TOF MS analysis showed that the missing protein spots were Rubisco large subunit. Subsequently, results of MALDI-TOF MS analysis of a major 55-kDa protein band from a 1-D sodium dodecyl sulfate-PAGE gel that disappeared from diseased tissues also showed that the protein was Rubisco large subunit. Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) is the predominant protein in leaves. Rubisco catalyzes the carboxylation of ribulose 1,5-bisphosphate (RuBP) to produce two molecules of 3-phosphoglycerate (3-PGA). Rubisco is composed of eight large (~56 kDa) and eight small subunits (~14 kDa). Western blotting of protein samples from healthy and diseased tissues using anti-Rubisco large subunit antibody confirmed that the protein was indeed the Rubisco large subunit (Fig. 2C). It has been shown in oat that the P protein of the glycine decarboxylase complex (GDC) binds to Victorin toxin (Wolpert and Macko, 1989; Wolpert et al., 1994). We therefore investigated if steady state levels of P

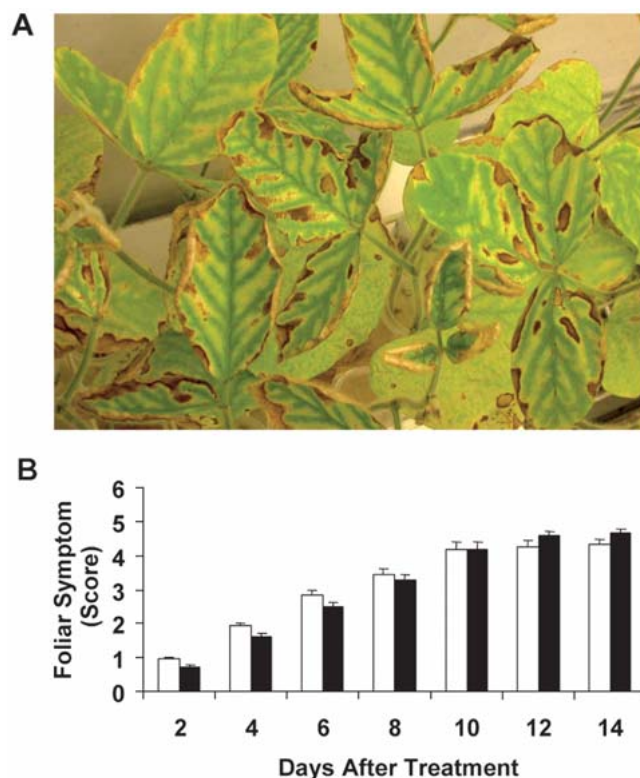


Fig. 1 SDS foliar symptom development in soybean seedlings fed with cell-free *Fsg* culture filtrate. (A) Foliar symptom development 14 days after treatment of cut soybean seedlings with the cell-free *Fsg* culture filtrate prepared from the *Fsg* isolate Clinton. Culture filtrate (1:24 dilution) was fed through cut ends of 3-week-old seedlings. (B) Foliar SDS symptom development following treating of seedlings with cell-free *Fsg* culture filtrates prepared from two *Fsg* isolates: Clinton (black bar) and Scott (white bar). Foliar symptoms were evaluated as follows: 0, no symptoms; 1, chlorotic spots; 2, interveinal chlorosis and necrotic spots (>30% of leaf area affected); 3, interveinal chlorosis (>50% of leaf area affected); 4, interveinal necrosis (>70% of leaf area affected); 5, severe interveinal necrosis (>90% of leaf area affected). No symptoms were observed when MSM was fed. Similar results were obtained in three independent experiments.

protein were also altered in diseased leaves by carrying out Western blot analysis. We observed that P protein of GDC was stable in diseased tissues (Fig. 2C).

Light accelerates Rubisco large subunit degradation

Light has been shown to be essential for development of toxin-induced disease symptoms (Daub and Briggs, 1983; Heiser et al., 1998, and references therein). We therefore investigated if light was essential for development of cell-free *Fsg* culture filtrate-induced leaf scorch in soybean. We observed that development of leaf scorch was not initiated when cell-free *Fsg* culture filtrate-fed soybean seedlings were incubated under dark conditions. This observation led us to investigate if absence of leaf scorch development in the dark was associated with the absence of Rubisco large subunit disappearance. We observed that, in the dark, levels of Rubisco large subunit did not change in the *Fsg* culture filtrate-fed seedlings. Exposure of the cell-free *Fsg* culture filtrate-fed seedlings to light, however, resulted in the disappearance of Rubisco large subunit and develop-

ment of leaf scorch symptoms (Fig. 3). Rubisco large subunit completely disappeared within 24 h of exposure of the cell-free *Fsg* culture filtrate-fed seedlings to light. Rubisco is a fairly stable enzyme (Peterson et al., 1973). We, therefore, concluded that disappearance of Rubisco large subunit was a result of its degradation rather than inhibition of its synthesis.

Light is essential for accumulation of free radicals in Fsg culture filtrate-fed leaves

The sudden loss of Rubisco in light-exposed, *Fsg* culture filtrate-fed seedlings led to failure of conversion of light energy into carbohydrates, and reducing equivalents produced in the light reaction, which were not utilized in carbon fixation. This presumably led to accumulation of free radicals, causing senescence-like cell death. We therefore hypothesized that, in absence of light, the extent of free radical accumulation in leaves of *Fsg* culture filtrate-fed seedlings should be much less than that in light-exposed leaves of *Fsg* culture filtrate-fed seedlings. We observed that exposure of cell-free *Fsg* culture filtrate-fed seedlings to light resulted in accumulation of free radicals (Fig. 4). In the absence of light, the cell-free *Fsg* culture filtrate failed to initiate the accumulation of free radicals to the levels produced in the presence of light.

In situ cell death in diseased leaves

In plants, programmed cell death (PCD), the equivalent of apoptosis in mammals, is a cell suicide process. The characteristics of PCD include cell shrinkage, chromatin condensation, DNA fragmentation, and appearance of apoptotic bodies (Greenberg, 1996). PCD has been suggested to be a mechanism for symptom development in many plant diseases (Yao et al., 2001; Lincoln et al., 2002). We therefore investigated whether PCD is observed during leaf scorch development. Endonucleolysis is the key biochemical event in apoptosis. We used TUNEL stain to detect any DNA fragmentation in nuclei. At the initial stage, PCD is characterized by fragmentation of nuclear DNA to approximately 50 kb. Labelled nucleotides were transferred to the free 3'-OH ends of oligonucleosomal DNA fragments *in situ* using terminal deoxynucleotidyl transferase (TdT) in a template-independent manner. This technique allows the labelling of broken DNA ends of only those nuclei that undergo programmed cell death. Fig. 5 shows DNA end labelling in leaf tissues from seedlings that were fed with the cell-free *Fsg* culture filtrate (Fig. 5F), but not in the leaf tissues fed with culture medium (Fig. 5B).

Discussion

Cell-free *Fsg* culture filtrate contains phytotoxins that cause foliar SDS (Li et al., 1999). We have observed that active components of cell-free *Fsg* culture filtrate causing foliar SDS bind to an anion exchange column suggesting that the active principle is negatively charged (data not shown). The 17 kDa, proteinaceous toxin of *Fsg* has been reported to also be negatively charged and was shown to cause browning of calli and necrosis of detached leaves in soybean (Jin et al., 1996).

Foliar symptoms developed following feeding of soybean seedlings with cell-free *Fsg* culture filtrates include chlorophyll loss and degradation of Rubisco, which are two hallmarks of senescence (Greenberg, 1996; Navarre and Wolpert,

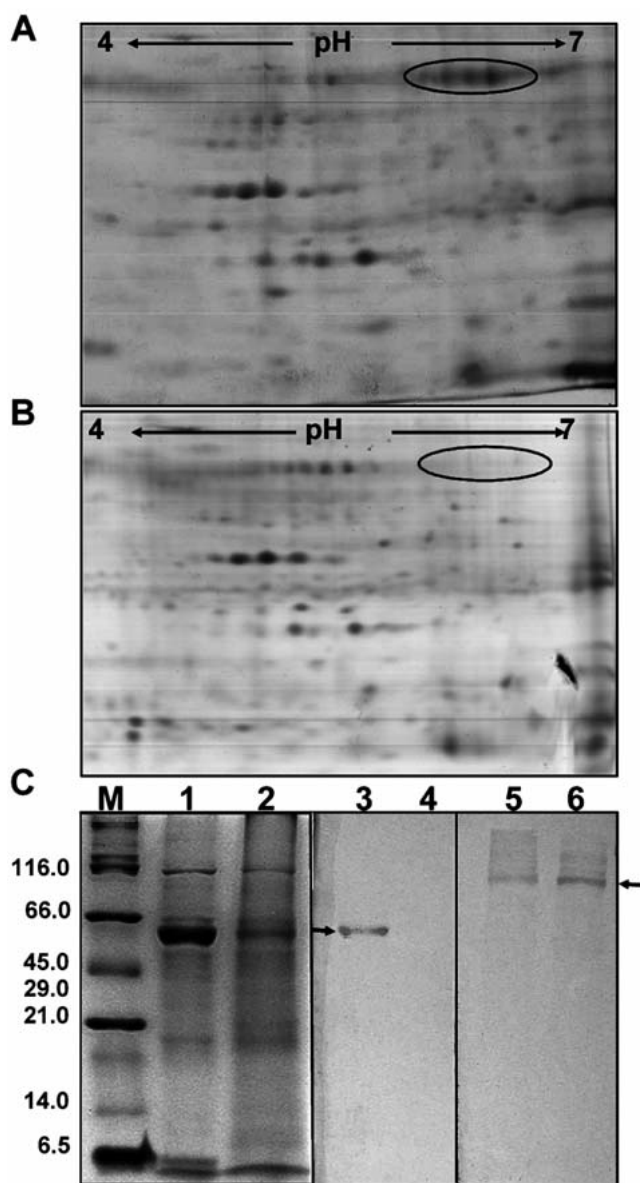


Fig. 2 Disappearance of Rubisco large subunit in diseased leaf tissues. (A) Two-dimensional (2-D) sodium dodecyl sulfate-PAGE of proteins prepared from healthy leaf tissues. (B) 2-D sodium dodecyl sulfate-PAGE of proteins prepared from diseased leaf tissues showing moderate symptoms (interveinal chlorosis and necrosis). Gels were stained with Coomassie Blue R250. The degraded protein spots are marked with a circle. The protein spots in the circle were identified as large subunits of Rubisco by MALDI-MS analyses. (C) Western blot analysis of the soybean proteins isolated from diseased and healthy tissues. Lanes 1, 3, and 5, proteins prepared from control healthy leaf tissues; lanes 2, 4, and 6, proteins prepared from diseased leaf tissues (interveinal chlorosis); lanes 1 and 2, Coomassie blue-stained sodium dodecyl sulfate-PAGE gel; lanes 3 and 4, Western blot of Rubisco large subunit; lanes 5 and 6, Western blot of the GDC-P protein. In each lane, 15 µg of total protein were separated. M, protein marker in kDa. Similar results were obtained from an additional experiment.

1999). Rubisco degradation is associated with two important physiological processes: senescence and plant responses to environmental stresses. During senescence, Rubisco, the most abundant leaf protein, provides amino acids to reproductive

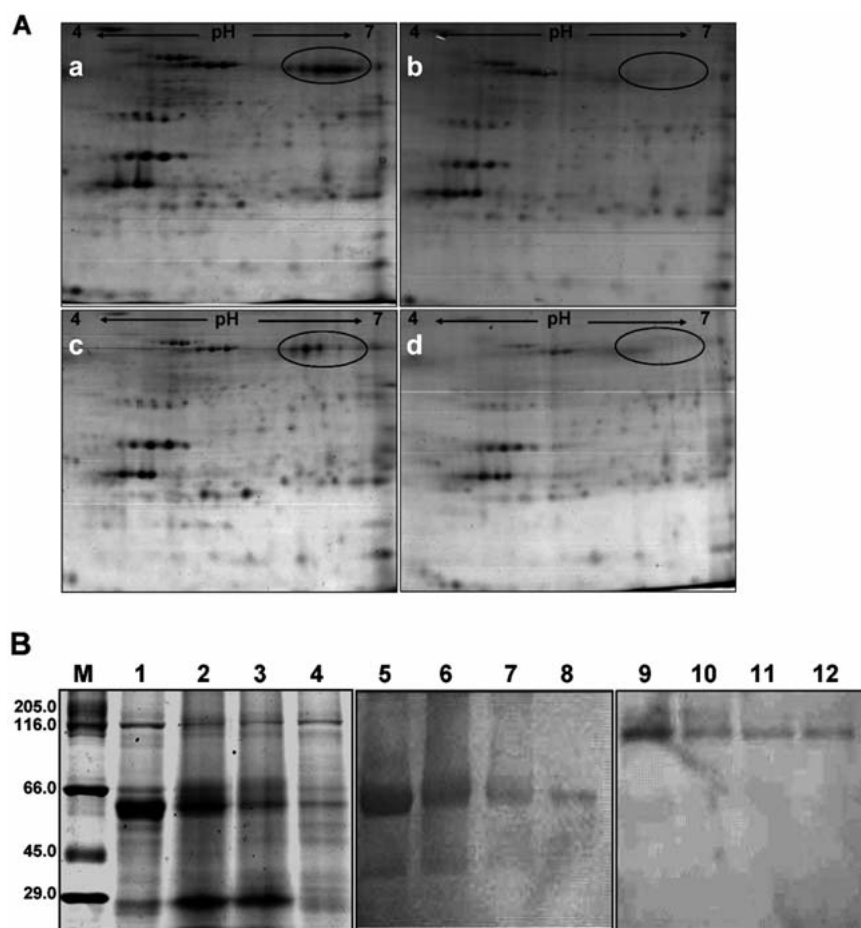


Fig. 3 Light is required for the disappearance of Rubisco large subunit by the *Fsg* culture filtrates. **(A)** 2-D sodium dodecyl sulfate-PAGE. The seedlings were fed with either cell-free *Fsg* culture filtrates or MSM through cut ends for 12 h and then exposed to light. Following light exposure, seedlings were returned to darkness for the remaining time of a total 36-h period. **a** Leaf proteins from seedlings exposed to light for 0 h following feeding with cell-free *Fsg* culture filtrate; **b** leaf proteins from seedlings exposed to light for 1 h following feeding with cell-free *Fsg* culture filtrate for 12 h; **c** leaf proteins from seedlings exposed to light for 8 h following feeding with culture medium only for 12 h; **d** leaf proteins from seedlings exposed to light for 8 h following feeding with cell-free *Fsg* culture filtrate for 12 h. The location of Rubisco large subunit is shown by circles. **(B)** Western blot analysis of the soybean leaf proteins isolated from dark- and light-exposed *Fsg* culture filtrate-fed

seedlings. Experimental design is similar to that of Fig. 3A. Lanes 1, 5, and 9: leaf proteins from seedlings exposed to light for 0 h following feeding with cell-free *Fsg* culture filtrate; lanes 2, 6, and 10: leaf proteins from seedlings exposed to light for 1 h following feeding with cell free *Fsg* culture filtrate for 12 h; lanes 3, 7, and 11: leaf proteins from seedlings exposed to light for 4 h following feeding with cell-free *Fsg* culture filtrate for 12 h; lanes 4, 8, and 12: leaf proteins from seedlings exposed to light for 8 h following feeding with cell-free *Fsg* culture filtrate for 12 h. Following light treatment, seedlings were returned to darkness for the remaining time of a total 36-h incubation period. Lanes 1–4: Coomassie blue-stained sodium dodecyl sulfate gel; lanes 5–8: Western blot of Rubisco large subunit; lanes 9–12: Western blot of the GDC-P protein; lanes 1–12, about 15 µg protein were separated in each lane. M, protein markers in kDa.

organs (Peoples et al., 1980; Makino et al., 1984; Ferreira and Teixeira, 1992). Environmental stresses such as low CO₂, oxidative stress, and toxin treatments can also induce Rubisco degradation (Ferreira and Davies, 1989; Casano and Trippi, 1992; Navarre and Wolpert, 1999). Interestingly, ROS are the common products generated during senescence and environmental stress (Desimone et al., 1996).

How the active principle of the *Fsg* culture initiates the degradation of Rubisco is yet to be uncovered. We have shown that light is essential for Rubisco degradation and accumulation of free radicals. The degradation of Rubisco presumably disrupts electron transport in chloroplasts. ATP and NADPH generated from photophosphorylation in chloroplasts are utilized by Rubisco for carbohydrate assimilation. In *Fsg* culture-fed leaves,

ATP and NADPH are not utilized due to degradation of Rubisco. As a result, ADP and NADP⁺ are not released for further photophosphorylation, leading to transfer of electrons from photosystem I to molecular oxygen and accumulation of ROS. Free radicals, produced in the presence of light presumably result in damage to leaf tissues, including initiation of PCD. The TUNEL assay showed nuclear DNA fragmentation in diseased leaves, an important characteristic of apoptosis. Similar results were observed in response to application of other host-selective toxins. For example, victorin causes oxidative damage in light-incubated oat tissues (Navarre and Wolpert, 1999). Host-selective and chlorophyllin toxins such as AAL and T toxins also require light for inducing necrosis (Moussatos et al., 1993; Bhullar et al., 1975).

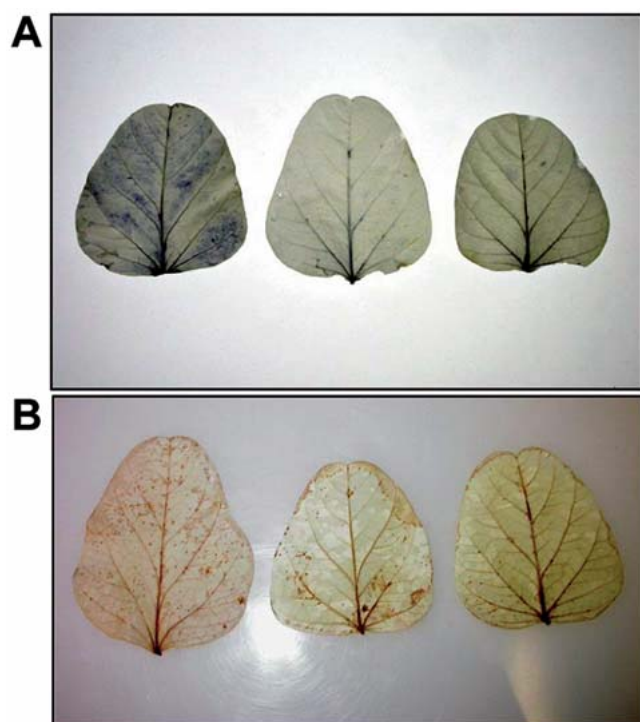


Fig. 4 *In situ* O_2^- and H_2O_2 accumulation in light-exposed *Fsg* culture filtrate-fed seedlings. Leaflets on the left are from cell-free *Fsg* culture filtrate-fed seedlings exposed to 1 h light. Leaflets in the middle are from cell-free *Fsg* culture filtrate-fed seedlings exposed to 0 h light. Leaflets on the right are from MSM-fed seedlings exposed to light for 1 h. The seedlings were fed with cell-free *Fsg* culture filtrates or MSM for 15 h in the dark prior to exposure to light. Following light exposure, leaves were immediately used for nitroblue tetrazolium or 3,3'-diaminobenzidine tetrahydrochloride assays. **(A)** *In situ* O_2^- identification by nitroblue tetrazolium assay. The blue colour indicates the O_2^- accumulation. **(B)** *In situ* H_2O_2 identification by 3,3'-diaminobenzidine tetrahydrochloride assay. The brown spot indicates accumulation of H_2O_2 . Similar results were obtained from two additional experiments.

Rubisco initiates the oxidative photosynthetic carbon pathway that requires cooperative interaction between chloroplasts, peroxisomes, and mitochondria. Rubisco catalyzes the oxygenation of ribulose 1,5-bisphosphate in chloroplasts. Glycine decarboxylase complex (GDC) is the key enzyme of this photorespiration pathway in mitochondria. Along with serine hydroxymethyl transferase, GDC metabolizes glycine into serine to end the photorespiration pathway. The four proteins that constitute GDC are P protein (100 kDa), H protein (15 kDa), T protein (45 kDa), and L protein (61 kDa). Victorin binds to the P protein of GDC and also to Rubisco (Wolpert and Macko, 1989; Navarre and Wolpert, 1995; Curtis and Wolpert, 2004). Our Western blot results showed that the GDC P protein was stable in diseased tissues. It is yet to be investigated if *Fsg* toxins cause mitochondrial dysfunction, as observed in oat leaves following Victorin treatments (Wolpert et al., 2002).

There are several similarities between physiological changes that occur in oat and soybean following exposure to Victorin and *Fsg* phytotoxins, respectively. They are: (i) degradation of Rubisco large subunit; (ii) accumulation of free radicals under light conditions; (iii) initiation of programmed cell death; and

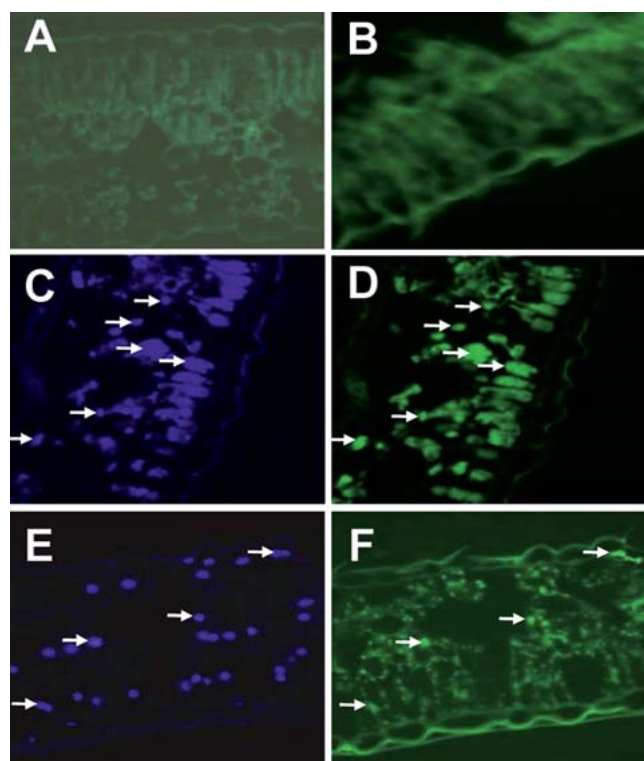


Fig. 5 DNA fragmentation in leaf tissues in response to exposure of seedlings to cell-free *Fsg* culture filtrates. **(A)** Leaf tissues were treated with TUNEL reaction solution without terminal deoxynucleotidyl transferase (TdT). **(B–F)** Leaf tissues were treated with TUNEL reaction solution containing TdT. Broken DNA ends in **D, F** were localized by labeling with the TUNEL reaction. Green spots shown by arrows are nuclei containing fragmented DNA. Blue spots represent nuclei stained with Hoechst 33258 dye in **C, E**. Staining of nuclei with Hoechst 33258 dye was conducted following the TUNEL reaction. **A–D** show leaf tissues from seedlings fed with MSM. Tissues shown in **C, D** were treated with DNAase before the TUNEL reaction; tissues shown in **E, F** are from seedlings fed with cell-free *Fsg* culture filtrates. Note that DNAase treatment of a MSM-fed leaf in **D** resulted in nuclei with broken DNA fragments localized by TUNEL reagents (shown by arrows). Without DNAase treatment, no such nuclei were observed in an MSM-fed leaf (**B**). Whereas, in the leaf of a seedling fed with the cell-free *Fsg* culture filtrate, nuclei contain broken or fragmented DNA labeled with TUNEL reagents, shown by arrows in **F**. Locations of the TUNEL-positive nuclei in **F** are shown in **E** with arrows. Similar results were obtained in an additional experiment.

(iv) necrosis in leaves. Therefore, it is most likely that both toxins cause diseases through a common mechanism. We are currently purifying the *Fsg* phytotoxin(s) in order to identify the *Fsg* toxin binding host protein(s) and will attempt to discover the mechanism by which the Rubisco degradation pathway and SDS development are initiated in soybean.

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